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RAPID DETERMINATION OF SOME PLASMA OXYPURINES USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

Human plasma levels of the endogenous oxypurines, xanthine and hypoxanthine, were measured using high-pressure liquid chromatography. Silicic acid columns were used to give high-resolution separations sensitive to 0.25 μg/ml using only 30 μl of ultrafiltered plasma. Enzyme treatment was used to establish oxypurine identities and freedom from interfering plasma components. Results and methodology are compared to previous work.

INTRODUCTION

High-pressure liquid chromatography is a procedure which offers rapid, high-resolution separations. Its application to the direct analysis of biological fluids has been largely limited to gradient elution on ion-exchange columns[1].

This paper describes an alternate method of analysis for ultraviolet (UV) absorbing materials in plasma using high-pressure chromatography and possessing a number of advantages over previously described ion-exchange systems.

It was our objective to develop a rapid, simple analysis for a variety of UV absorbing compounds in human blood plasma. Ion-exchange chromatography immediately suggested itself, but commercially available (Reeve Angel Co., Varian Aerograph) pellicular anion-exchange resins were expensive and found to give low efficiency without gradient elution. Gradient elution, however, requires the use of an additional constant-volume displacement pump and a reequilibration period for the column after each injection. Silicic acid columns, by contrast, are inexpensive, give high efficiency without gradient elution and are stable over long periods of time. The method described uses silicic acid columns to give high-resolution separations sensitive to 0.25 μg/ml using only 30 μl of plasma. No complicated extractions or other pretreatments are necessary, only a simple deproteinization.

METHODS

Instrumentation

A Varian Aerograph Model 4100 high-pressure liquid chromatography system

was used. It consists of a pump capable of delivering 5000 p.s.i. at constant flow-rates up to 200 ml/h, a constant-temperature water-bath regulating to $\pm$ 0.01°, and a sensitive UV detector which reads to 0.005 *A* full scale at 254 nm. The detector output was recorded on a Varian Aerograph A-25 recorder. A Whitey three-way valve was used to depressurize the column before injection.

One major difficulty encountered was the formation of gas bubbles in the detector. This was effectively overcome by forcing the detector effluent to the bottom of a 30-cm column of mercury (Fig. 1). This device eliminated the need for solvent degassing, and prevented the vaporization of an ether based solvent at 55°. The detector is rated to a maximum back pressure of 1 atm, so the mercury column could presumably have a maximum height of 76 cm.

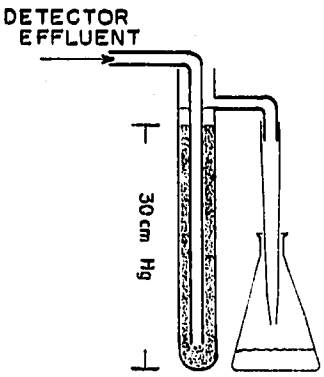


Fig. 1. Simple device to prevent the vaporization of low-boiling solvents in an UV detector. Constant pressure is maintained regardless of flow-rate.

The signal-to-noise ratio of the detector was improved by a factor of better than ten by completely wrapping it in glass-wool to protect it from air currents.

Materials

All chemicals used were A.R. grade (Mallinckrodt Chemical Works). The ethyl ether contained approx. 2% ethanol and 0.5% water as stabilizer. Xanthine and hypoxanthine were Grade A from Calbiochem, Inc.

Xanthine oxidase was purchased from Mann Research. It was freed from low-molecular-weight contaminants in the following manner: 0.5 ml of a 10-mg/ml solution of xanthine oxidase was placed in a Centriflo ultrafiltration membrane cone 2100 CF50 (Amicon Corporation). The filter cone was centrifuged at 1000 $\times$ *g* for 30 min at 10°. The filtrant was reconstituted with 0.5 ml of 20% $(NH_4)_2SO_4$ and centrifuged again. This process was repeated three times and produced an enzyme solution greatly reduced in UV absorbing contaminants. Enzyme activity loss after this treatment was not measured, but a sufficient amount was retained for the experiment described.

Chromatography columns

Tubing, 316 s.s., 0.125 in. O.D., 0.055 in. I.D. $\times$ 3 m was purchased from Tube Sales Company, Los Angeles, Calif. It was packed with Bio-Sil A silicic acid, 25–35 μ, purchased from Bio-Rad Laboratories. Packing was done by adding the silicic acid

powder in small aliquots of approx. 300 mg to the top of the column, and then tapping lightly on the side while simultaneously tapping the column vertically against the floor. A column of maximum efficiency was obtained allowing 45 sec between adding aliquots. A column terminator with a 2-μ replaceable filter was purchased from Varian Aerograph.

A 3-in. precolumn was packed with the same material except for the very top which was packed with about 1/8 in. of glass-wool. The glass-wool prevented blow-back of packing material into the syringe while making an injection. The 3-in. pre-column effectively trapped the residual protein and other eluent insoluble materials from the plasma, and was replaced when the pump delivery pressures became excessive. The lifetime of a precolumn while running approx. 8 plasma samples per day was about 2 weeks. The lifetime of the main column is well in excess of 2 months of continuous use, retention volumes of the compounds remain reproducible and the standard curves remain valid.

Chromatography

An aqueous solution of 30 μl was injected on the 3-m silicic acid column previously described. The elution pattern of some oxypurines normally found in plasma and some common drugs is illustrated in Fig. 2a. The solvent system used was ethyl ether–*n*-propanol–5% acetic acid in water (35:14:4) flowing at 45 ml/h.

Ethyl ether was used because of its very low viscosity, and *n*-propanol because of its strong elution properties and desirable plasma separation characteristics.

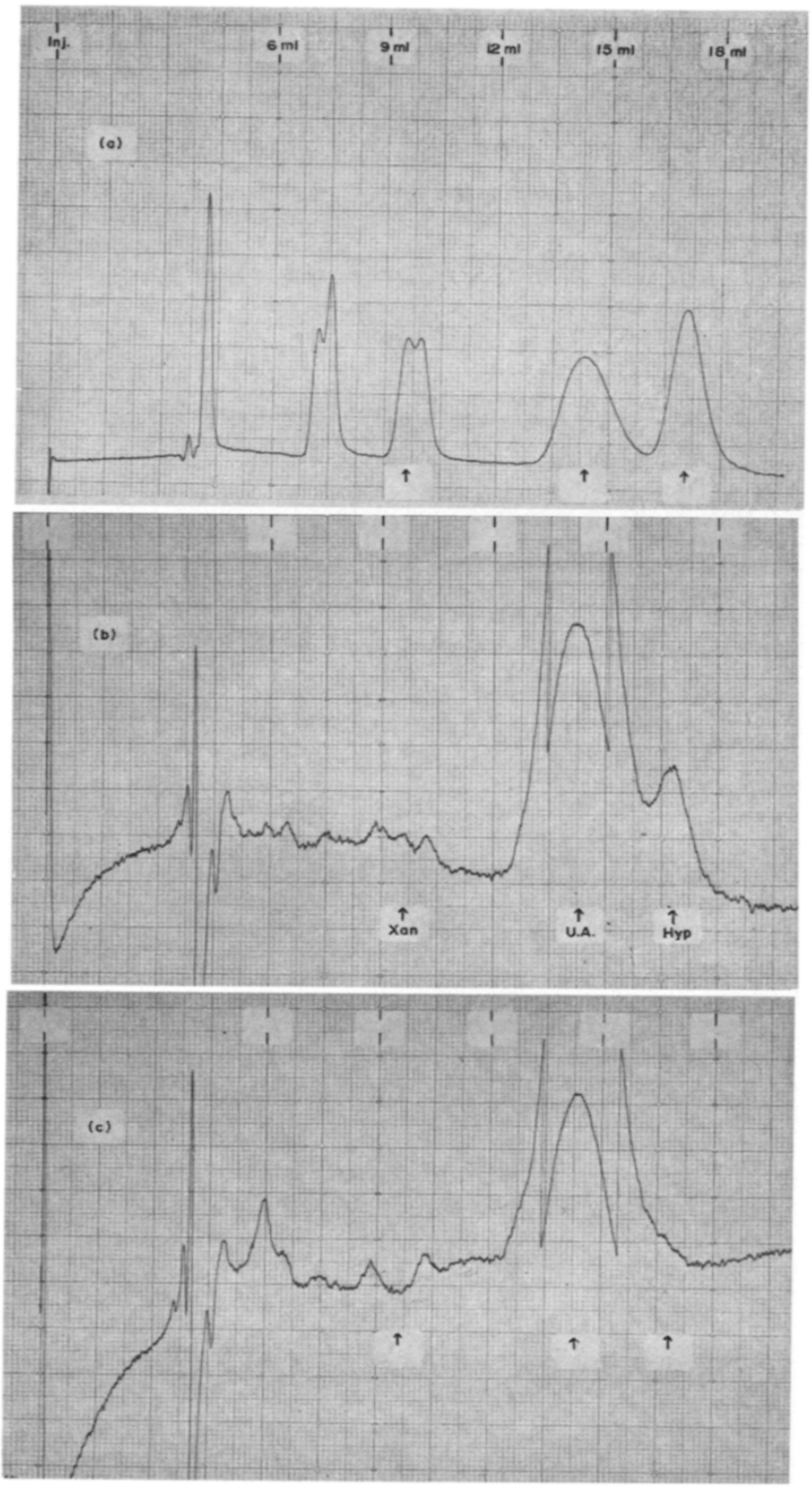
Water concentration was a compromise between the relative uric acid retention volume, column back pressure, and column efficiency. Increasing water concentration reduced the retention volume of uric acid relative to hypoxanthine and xanthine, increased back pressure and increased column efficiency. Decreasing water concentration had the opposite effects.

The small amount of acetic acid was necessary to ensure reproducible retention volumes between aqueous standards and deproteinized plasma samples. Other solvent systems with no acetic acid led to variable retention volumes and irreversible pressure build-up within a few days of use.

Micro-technique for human blood plasma sampling

Since it is only necessary to use 30 μl of deproteinized plasma for an analysis, milliliter quantities of blood need not be drawn.

A finger puncture was sufficient to fill a chilled, heparinized micro blood collecting tube holding 370 μl (Scientific Products). The small end of the tube was sealed with critoseal (Scientific Products), and wrapped with tape. The tube was then centrifuged at 5° in a table top centrifuge for 15 min, taken out and carefully broken at the juncture between the plasma and blood cells. Using a light coating of stopcock grease at the breakage point prevented the plasma from wetting the outside of the tube and running out. The plasma was blown into an ultrafiltration membrane cone previously described. The ultrafiltration was carried out at 1000 $\times$ g for 45 min at 10°, and about 60 μl of a clear ultrafiltrate was obtained. The entire process was carried out as rapidly as possible.


Inj.
6 ml
9 ml
12 ml
15 ml
18 ml
(a)
(b)
Xan
U.A.
Hyp
(c)

VALIDATION OF THE METHOD

As an example of the usefulness of the method in plasma, quantitative determinations of two endogenous oxypurines, hypoxanthine and xanthine, were made.

5 ml of blood from human volunteers was withdrawn into a chilled, heparinized vacutainer, the blood cells were immediately removed by centrifugation and the plasma stored at −5°.

200 μl of the thawed plasma was placed on a Centriflo ultrafiltration membrane cone 2100 CF50 and centrifuged at 1000 × *g* for 45 min at 10°. The ultrafiltrate (30 μl) was injected on the silicic acid column under the conditions previously described and the resultant chromatogram is pictured in Fig. 2b. 50 μl of the same ultrafiltrate was reacted with 5 μl of the purified xanthine oxidase solution for 30 min at room temperature. The expected effect is the oxidation of xanthine and hypoxanthine to uric acid, and this is observed in Fig. 2c.

TABLE I

RECOVERY OF XANTHINE AND HYPOXANTHINE ADDED TO PLASMA

Addition	*Plasma sample (No.)*					*Mean ± S.D. (μg/ml)*
	1	*2*	*3*	*4*	*5*	
Xanthine, 1.08 μg/ml						
before addition	0.19	0.51	0.0	0.26	0.0	
after addition	1.17	1.49	1.0	1.55	1.06	
difference	0.98	0.98	1.0	1.29	1.06	1.06 ± 0.13 98% ± 12%
Xanthine, 2.38 μg/ml						
before addition	0.17	0.41	0.0	0.23	0.0	
after addition	2.45	2.83	2.23	2.83	2.06	
difference	2.28	2.42	2.23	2.60	2.06	2.32 ± 0.20 97% ± 9%
Hypoxanthine, 1.17 μg/ml						
before addition	1.19	2.0	0.0	0.82	0.09	
after addition	2.25	2.94	0.93	2.07	1.09	
difference	1.06	0.94	0.93	1.25	1.0	1.04 ± 0.12 89% ± 12%
Hypoxanthine, 2.57 μg/ml						
before addition	1.05	1.76	0.0	0.72	0.08	
after addition	3.61	3.82	2.16	3.82	2.22	
difference	2.56	2.06	2.16	2.10	2.16	2.21 ± 0.20 86% ± 9%

Fig. 2. Elution pattern. (a) 30 μl of approx. 10 μg/ml each of (left to right) salicylic acid (4.2 ml elution volume), theophylline (7.2 ml), *allo*purinol (7.5 ml), xanthine (9.6 ml), caffein (10 ml), uric acid (14.3 ml), and hypoxanthine (17.1 ml). Column and solvent system used were as described in text; flow-rate, 45 ml/h. Column temperature, 54°. Pressure, 4600 p.s.i. Detector attenuation, 0.08 *A* full scale. (b) 30 μl of normal human plasma ultrafiltrate, showing about 0.2 μg/ml xanthine and 1.37 μg/ml hypoxanthine. Same conditions as above except full scale attenuation of 0.01 *A*. (c) Same sample as (b) but treated with purified xanthine oxidase.

For recovery studies, 0.5–1 μg of xanthine and hypoxanthine were added to 0.5 ml of normal plasma. 200 μl of this plasma solution was ultrafiltered in the manner described and then analyzed. The results are given in Table I with correction for endogenous amounts of hypoxanthine and xanthine. The accuracy obtained from an actual analysis would be somewhat better, since the recoveries in Table I are the results of two analyses, one for endogenous and one for added plus endogenous material, and the error is thereby increased.

Standard curves for hypoxanthine and xanthine are illustrated in Fig. 3, with a least squares fit for the best straight line.

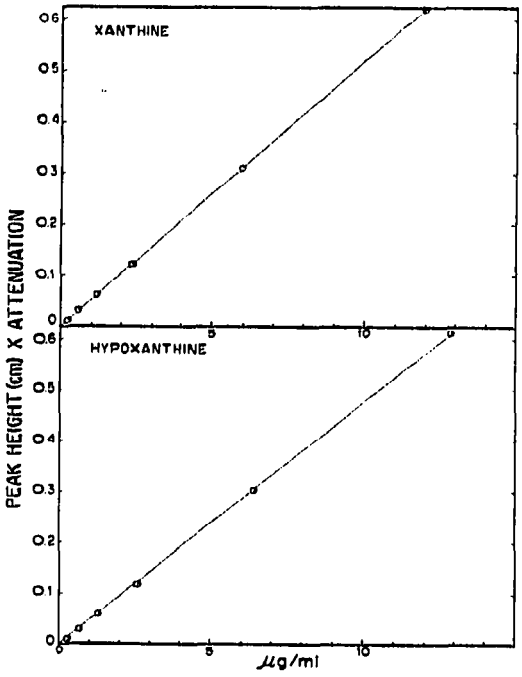


Fig. 3. Standard curves for aqueous solutions of hypoxanthine and xanthine using 30-μl injections. Chromatography conditions are identical with those in Fig. 2 and as described in text. Xanthine: slope = 0.0521, y intercept = −0.001. Hypoxanthine: slope = 0.0476, y intercept = −0.0017.

RESULTS AND DISCUSSION

Normal human plasma levels of the oxypurines, hypoxanthine and xanthine, were determined using the micro-blood sampling technique. All subjects were adults and had fasted for 12 h prior to sample taking. Results are shown in Table II.

Literature values for normal hypoxanthine and xanthine plasma levels vary widely and are given in Table III. Orsulak *et al.*[2] measured oxypurines from plasma by a simple thin-layer chromatographic separation, elution and direct spectrophotometric quantitation. Recovery studies were done, but no confirmation as to the identity of the substances was given. Sweetman and Nyhan[4] described a method using cation-exchange column chromatography, but gave no normal levels for plasma.

TABLE II

NORMAL LEVELS OF HYPOXANTHINE AND XANTHINE IN HUMAN PLASMA

Subject	*Xanthine (μg/ml)*	*Hypoxanthine (μg/ml)*
Male		
G.F.	<0.25	0.42
E.P.	<0.25	1.49
B.S.	<0.25	0.53
J.B.	<0.25	0.59
A.G.	<0.25	0.73
Female		
E.F.	<0.25	0.26
F.M.	<0.25	0.34
M.A.	<0.25	<0.25

TABLE III

LITERATURE VALUES FOR NORMAL PLASMA OXYPURINE LEVELS

Ref.	*Xanthine (μg/ml)*	*Hypoxanthine (μg/ml)*	*No. of subjects*
2	4.5 ± 0.3	5.2 ± 0.3	6
3	5.2 ± 0.6	6.0 ± 0.3	15
4	—	—	0
5	0.27	0.081	1
6	0.8 – 2.1	0.8 – 1.7	12
7	0 – 1.3	1.1 – 1.8	5
8	0.7 – 1.2	0.6 – 1.1	not given
This study	<0.25	Men 0.42 – 1.49	5
		Women <0.25 – 0.34	3

Chalmers and Watts[5] used an enzymatic differential spectrophotometric method and reported the results from one subject on an unrestricted diet.

Hayashi and Gilling[6] used a relatively complicated assay procedure employing 15–20 ml of plasma, Sephadex deproteinization, ^{14}C-labeled oxypurines, anion-exchange, and an enzymatic spectrophotometric measurement. Snedden and Parker[7] used quantitative high-resolution mass spectrometry to determine the oxypurines in plasma and tissues. Müller[8] has recently reviewed the subject and gave normal oxypurine values for plasma using a modification of Orsulak *et al.*[2].

The results of this study compare well with those of Snedden and Parker[7], as well as with most determinations of hypoxanthine plus xanthine. Measured in terms of hypoxanthine, Klinenberg *et al.*[9] found normal plasma oxypurine levels of 0.65–1.65 μg/ml, Chalmers and Watts[10] of 0.32 μg/ml, and Segal and Wyngaarden[11] found normal levels of 0–2.5 μg/ml.

Other compounds of widely varying chemical nature have also been well quantitated in this system, and show the same high degree of sensitivity and accuracy as illustrated by xanthine and hypoxanthine. Uric acid can also be determined under these chromatographic conditions, although it is an exceptionally broad peak and

exhibits some tailing. If desired, it can be removed by uricase before ultrafiltration, making hypoxanthine quantitation much easier.

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REFERENCES

1 C. D. Scott, R. L. Jolley, W. W. Pitt and W. F. Johnson, *Amer. J. Clin. Pathol.*, 53 (1970) 701.
2 P. J. Orsulak, W. Haab and M. D. Appleton, *Anal. Biochem.*, 23 (1968) 156.
3 M. D. Appleton, W. Haab, U. Burti and P. J. Orsulak, *Amer. J. Ment. Defic.*, 74 (1969) 196.
4 L. Sweetman and W. L. Nyhan, *Anal. Biochem.*, 31 (1969) 358.
5 R. A. Chalmers and R. W. E. Watts, *Analyst (London)*, 94 (1969) 226.
6 T. T. Hayashi and B. Gilling, *Anal. Biochem.*, 36 (1970) 343.
7 W. Snedden and R. B. Parker, *Anal. Chem.*, 43 (1971) 1651.
8 M. M. Müller, *Wien. Klin. Wochenschr.*, 84 (1972) 206.
9 J. R. Klinenberg, S. Goldfinger, K. H. Bradley and J. E. Seegmiller, *Clin. Chem.*, 13 (1967) 834.
10 R. A. Chalmers and R. W. E. Watts, *Analyst (London)*, 93 (1968) 354.
11 S. Segal and J. B. Wyngaarden, *Proc. Soc. Exp. Biol. Med.*, 88 (1955) 342.